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Study on optimization for *in vitro* propagation of *Dalbergia oliveri* by plant tissue culture

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Abstract The experimental findings demonstrated that the highest proliferation frequency (100%), along with the maximum number of shoots per explant (3.33±0.52 shoots) and an average shoot length of 30.04±1.70 mm, were achieved in the Murashige and Skoog (MS) medium supplemented with 2.0 mg/l of 6-benzylaminopurine (BAP). Subsequently, upon reaching a height of 3 to 4 cm, the in vitro micro shoots were transferred to a ¼ MS medium enriched with 0.75 mg/l of indole-3-acetic acid (IAA). This particular regimen resulted in the highest number of roots per shootlet (3.50±0.58) with an average root length of 34.19±0.79 mm. The acclimatization phase of the plantlets to the soil was successful, demonstrating a commendable survival rate of 80%.

Keywords: Acclimatization, *Dalbergia oliveri*, *In vitro* micro shoots, Propagation

Introduction

Dalbergia oliveri Gamble ex Prain, commonly recognized as rosewood, pertains to the Fabaceae family.It is an economically significant plant distributed in evergreen tropical or semi-deciduous forests (Winfield *et al*., 2016). Rosewood is known for its hardness, heaviness, and beautiful reddish timber. Soaring to heights exceeding 20 meters, this tree species boasts an impressive stature, its strong trunk measuring a diameter of over 60 centimeters (Danida, 2004). It is extensively utilized in producing luxury furniture, instruments, decorations, wood carvings and construction activities (Winfield *et al*., 2016). Unfortunately, rosewood is frequently targeted in illegal harvesting and traded locally and globally (Barstow *et al*., 2022). As a result of overexploitation, the population of mature trees has experienced a significant decline. Although seed

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production in large-sized trees is typically sufficient, natural germination is often poor. Without protective measures, this species can face the risk of extinction. (Danida, 2004). Being officially recognized as a hongmu species, *Dalbergia oliveri* encounters substantial threats in its natural habitat as it bears the brunt of extensive timber harvesting. It is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). (Barstow *et al*., 2022). The *in vitro* culture technique is currently widely utilized to address issues related to the preservation and rejuvenation of the genetic material of rare plant species at risk of extinction, which is of paramount importance. As a result, the propagation of *D. oliveri* through *in vitro* culture can yield many new plants within a short period while occupying limited space and incurring low costs. This method presents an efficient means of rapidly multiplying the population of *D. oliveri*, thereby facilitating this species, conservation and restoration efforts. The objective was to progress an optimized protocol that facilitates *in vitro* micropropagation, rooting, and successful acclimatization.

Materials and methods

Surface sterilization

The healthy mature seeds of *Dalbergia oliveri* were thoroughly cleansed by rapid rinsing with tap water, a process that lasted for 10 minutes. They were then surface disinfected for 30 minutes using a 1% carbendazim solution. Following the surface disinfection, the seeds underwent 2 to 3 rinses using sterile distilled water within a laminar airflow. These seeds were subjected to additional sterilization following the washing and surface disinfection steps. They were soaked in a 5% (v/v) bleach solution, 0.1% (w/v) mercuric chloride solution ($HgCl_2$), a 0.1% (v/v) cefotaxime solution, and a 0.1% (v/v) plant preservative mixture (PPM) along with three drops of Tween 20 for 20 mins, and the seed was washed twice with sterile distilled water after a duration of 10 minutes. After the sterilization and rinsing steps, These seeds were germinated on MS (Murashige and Skoog, 1962) medium and WPM (Lloyd and McCown, 1981) at different concentrations (½ and 1) with 3.0% (w/v) sucrose, 0.1 % activated charcoal (AC) and 2.6 g/L of phytage (*Phyto*Technology Laboratories). The samples were placed in the controlled plant growth room, where they were kept at 25±2°C and subjected to a 16/8-hour (light/dark) photoperiod.

Induction of multiple shoots

The cotyledon node explants were derived from sterile seedlings that were 30 days old. These explants were then placed on MS (Murashige and Skoog) medium added with different concentrations (0.5, 1.0, 2.0, and 3.0 mg/l) of three different plant growth regulators: BAP (6-benzylaminopurine), *m*T (*meta*-Topolin), or GA_3 (Gibberellin). The samples were placed in the controlled plant growth room, where they were kept at 25±2°C and subjected to a 16/8-hour (light/dark) photoperiod. The cultures underwent regular transfers to a fresh medium, with the procedure being performed every 30 days. After 30 days on the medium, recordings were made for the average number of shoots per explant along with their respective lengths.

Rooting and acclimatization

The movement of healthy microshoots, measuring 3 to 4 cm in length, to ¼ MS and ½ MS media with different concentrations (0.5, 0.75, 1.0, and 2.0 mg/l) of IAA (indole-3-acetic acid). The samples were placed in the controlled plant growth room, where they were kept at 25±2°C and subjected to a 16/8-hour (light/dark) photoperiod. The cultures underwent regular transfers to a fresh medium, with the procedure being performed every 30 days. After 30 days on the medium, the average number of roots per shoot and the root length were recorded. The plantlets, boasting well-developed root systems, were cleansed by rapid rinsing with tap water to eliminate the growth medium. Afterwards, they were transplanted into pots filled with a sterile soil and perlite mixture at a ratio of 2:1. The pots were then covered with polyethylene bags to create a suitable microclimate. The plantlets were cultured under the same environmental conditions as before for 14 days to allow for further growth and acclimatization. After this period, the plantlets were ready for transfer to the greenhouse.

Statistical analysis

The experimental design was conducted using a completely randomized design. Three duplicates of 15 explants. The data underwent statistical analysis of variance (ANOVA), and mean separation was accomplished through Duncan's multiple range test ($P \leq 0.05$) using SPSS 17 software.

Results

The impact of basal medium on the in vitro germination of seeds

The highest germination rate (85.71%) was achieved using ½ MS media with 0.1% AC. The shorter time for germination was 20.75 days. On the contrary, when placed in MS, ½ WPM, and WPM media, the germination time was longer, and the germination rate was lower compared to the half-strength MS medium (Figure 1).

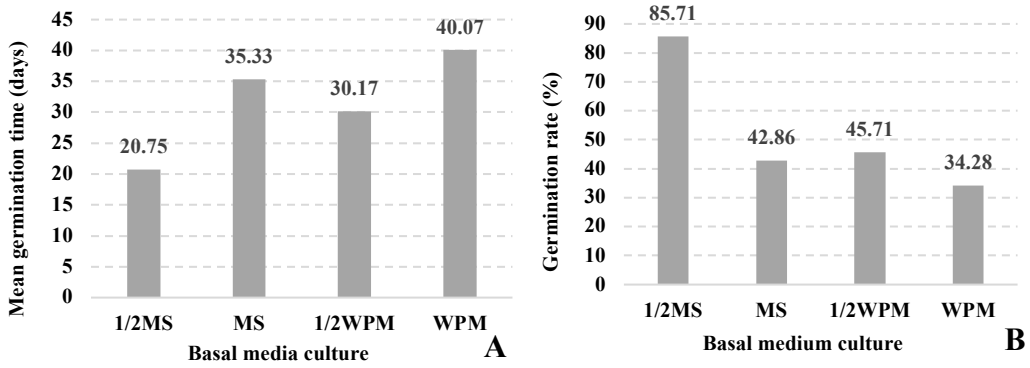

Figure 1. *In vitro* seed germination of *Dalbergia oliveri*. A) Mean germination time of *D. oliveri* when cultured on basal medium added with 0.1% AC. B) The germination rate of D. oliveri when cultivated on basal medium added with 0.1% AC

The initiation of multiple shoot formation from the cotyledon node

The experiment aimed to evaluate the initiation of multiple shoots using cotyledon node explants of *Dalbergia oliveri*. The cultures were carried out on an MS media added 30 mg/l sucrose, with various plant growth regulators (BAP, *mT*, or GA_3) applied at various concentrations (0.5, 1.0, 2.0, and 3 mg/l) for 4 weeks. The results revealed that shoot induction by BAP was superior to that achieved with *mT* and GA_3 (Table 1). Cotyledon fragments cultivated in the presence of BAP successfully induced healthy shoots with a green coloration and exhibited the maximum shoot length under the BAP treatments in all conditions (Figure 2). In comparison to other growth regulators in the experiment, it was observed that a concentration of 2 mg/l BAP led to the highest shoot response (100%), shoot length (30.04 mm) and an average of 3.33 shoots per explant. The experimentation utilizing MS media supplemented with 2.0 mg/l of GA_3 and 0.5 mg/l of *m*T resulted in the growth of 1.75 and 1.33 shoots per explant,

respectively, and an average shoot length of 43.00 mm and 17.93 mm. However, the observed response to shoot regeneration was notably low, with a success rate of only 30% for the respective concentrations (Table 1).

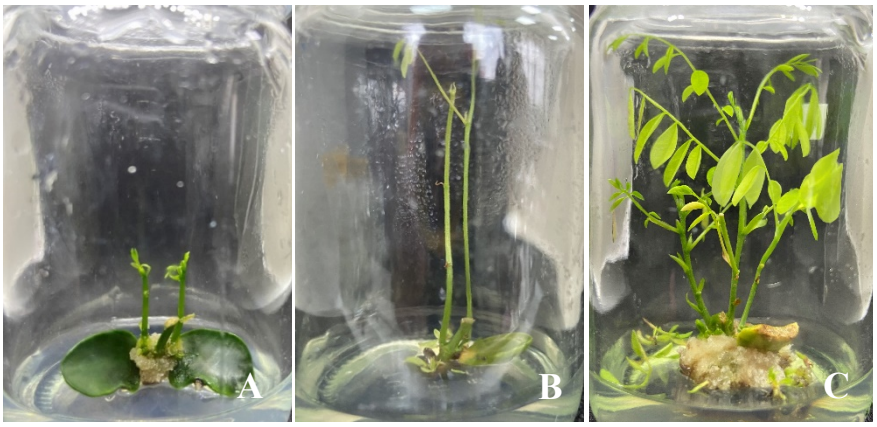

Figure 2. Multiple shoots developed from a cotyledonary node explant of *Dalbergia oliveri* after 30 days. A) Multiple shoots in cotyledonary node on MS+0.5 mg/l *m*T. B) Multiple shoots in cotyledonary node on MS+2.0 mg/l GA_3. C) Multiple shoots in cotyledonary node on MS+2.0 mg/l BAP

Table 1. The effects of different concentrations of 6-benzylaminopurine (BAP), *meta*-Topolin (*m*T), or Gibberellin (GA_3) the induction of multiple shoots from cotyledonary nodes of *Dalbergia oliveri*

Plant growth regulator (mg/l)			Shoot proliferation (%)[1/]	No. of shoot per explant[2/]	Shoot length (mm)
BAP	GA_3	*mT*			
0	0	0	-	-	-
0.5			60	2.00^{bc}±0.00	23.67^{d}±1.44
1.0			80	2.00^{bc}±0.00	24.35^{d}±1.06
2.0			100	3.33^{a}±0.52	30.04^{b}±1.70
3.0			100	2.33^{b}±0.52	26.28^{cd}±2.52
	0.5		20	1.00^{d}±0.00	28.37^{cd}±1.21
	1.0		30	1.33^{cd}±0.58	26.38^{cd}±0.96
	2.0		30	1.75^{bcd}±0.50	43.00^{a}±1.00
	3.0		20	1.67^{bcd}±0.58	26.30^{cd}±1.63
		0.5	0	1.33^{cd}±0.58	17.93^{e}±0.67
		1.0	30	1.50^{cd}±0.71	16.09^{e}±0.90
		2.0	-	-	-
		3.0	-	-	-

- no response

[a-f] In the same column, distinct letters signify statistically significant differences at $P < 0.05$, as determined by Duncan's Multiple Range Test (DMRT) following one-way ANOVA analysis.

Rooting and acclimatization

The microcuttings were translocated to media containing both ¼ MS and ½ MS formulations, each with various concentration (0.5, 0.75, 1.0, and 2.0 mg/l) of IAA (indole-3-acetic acid). The best rooting percentage reached 80% within 12-15 days after cultivation in ¼ MS medium supplemented with 0.75 mg/l IAA (Figure 3A). Similarly, IAA contributed to an increase in both the number of roots per shoot (3.50 roots) and the length of roots (34.19 mm) after 30 days in culture (Figure 3B). The plantlets were subsequently transplanted into pots filled with a sterile soil and perlite mixture at a 2:1 ratio. Polyethylene bags were utilized to cover these pots, and the plantlets were maintained under the same conditions for 14 days before being translocated to the greenhouse for acclimatization.

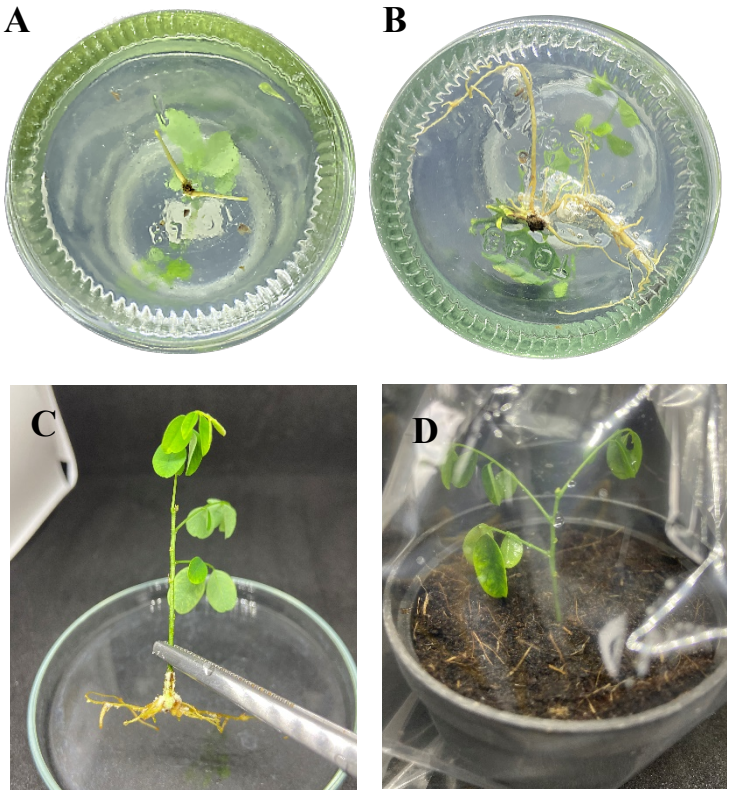

Figure 3. Rooting and acclimatization of *D. oliveri*. A) Rooting of *in vitro* developed microshoot on ¼ MS+ 0.75 mg/l IAA for 15 days. B) and 40 days. C, D) Plantlets cultivated entirely *in vitro* were transplanted into soil and acclimatized to *ex-vitro* conditions

Table 2. Effects of culture medium and IAA (indole-3-acetic acid) combination on root inductions from microshoot of *Dalbergia oliveri*

Plant growth regulator (mg/l)	Medium used	Root induction (%)[1/]	No. of roots per microshoot [2/]	Root length (mm) per microshoot
0	¼ MS	-	-	-
IAA 0.5	¼ MS	40	2.00^{bc}±0.00	17.38^{c}±1.81
IAA 0.75	¼ MS	80	3.50^{a}±0.58	34.19^{a}±0.79
IAA 1.0	¼ MS	40	2.50^{b}±0.71	8.50^{e}±0.85
IAA 2.0	¼ MS	-	-	-
0	½ MS	-	-	-
IAA 0.5	½ MS	40	1.00^{cd}±0.00	11.94^{d}±1.83
IAA 0.75	½ MS	40	1.50^{bc}±0.71	22.07^{b}±2.57
IAA 1.0	½ MS	-	-	-
IAA 2.0	½ MS	-	-	-

- no response

[a-f] In the same column, distinct letters signify statistically significant differences at $P < 0.05$, as determined by Duncan's Multiple Range Test (DMRT) following one-way ANOVA analysis.

Discussion

The cultivation of *Dalbergia oliveri* seeds was investigated using ½ MS, MS, ½ WPM, and WPM media, all supplemented with 30g/l sucrose, 0.1% activated charcoal (AC), and 2.6 g/L of phytage. The results revealed that using the half-strength MS medium resulted in the highest seed germination rate and the shortest seedling development duration. The ½ MS medium was well-suited for the seed growth of *B. monosperma* var. lutea (Pradhan *et al*., 1998; Yarra et *al*., 2016). Multiple shoots were initiated from cotyledonary node explants extracted from 3-week-old, *in vitro*-germinated seeds on an MS medium supplemented with BAP, *m*T, and GA_3 at concentrations of 0.5, 1.0, 2.0, and 3.0 mg/l. It was observed that BAP at a concentration of 2 mg/l was effective in inducing healthy shoots. As a result, this treatment led to the highest shoot growth percentage, reaching 100%, along with the most extended average shoot length of 30.04 mm and an average of 3 shoots per explant. The addition of BAP concentrations into the media, whether surpassing or falling below the optimal threshold, resulted in a diminution of the numbero of shoot. (Arya *et al*., 2013; Bhandari *et al*., 2021; Rathnaprabha *et al*., 2017; Vibha *et al*., 2014). However, cotyledons cultivated with GA_3, despite achieving a maximum shoot length, exhibited adverse effects such as hyperhydricity, a lack of leaves, and tip burning (Fráguas *et al*.,2004). *m*T was observed to stimulate the growth of solid shoots, marked by a dark green color and a tendency to produce a high number of shoots. However, a notable disadvantage is these shoots, stunted and incomplete nature, which makes them unsuitable for successful rooting induction. The most favorable rooting response was in ¼ MS media with 0.75 mg/l IAA. This resulted in an 80% rooting percentage, with an average of 3.50 roots per shoot and a root

length of 34.19 mm after 30 days. These roots displayed branching and elongation. The optimal rooting response utilizing IAA has been previously documented for leguminous trees, including *Albizzia chinensis* (Borthakur *et al*., 2012), and *Dalbergia cochinchinensis* (Supapas *et al*., 2022). The successful acclimatization of *Dalbergia oliveri* can be attributed to the stability of the cultivation area and the optimal humidity levels in the root system of the plants.

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References

Arya, I. D., Nautiyal, S. and Arya, S. (2013). Tissue culture studies on clonal variations in micropropagation of *Dalbergia sissoo*. International Journal of Biotechnology Research 1:058-070.

Bhandari1, A., Singh, H., Srivastava, A., Kumar, P., Panwar, G. S. and Mao, A. A. (2021). *In vitro* propagation and cytological analysis of *Sophora mollis* Royle: an endangered medicinal shrub. Journal of Genetic Engineering and Biotechnology 19:40.

Borthakur, A., Das, S. C., Kalita, M. C. and Sen, P. (2012). An *in vitro* plant regeneration system for conservation of the leguminous tree *Albizzia chinensis* (Osbeck) Merr. Pelagia Research Library, 3:1727-1732.

Barstow, M., Boshier, D., Bountithiponh, C., Changtragoon, S., Gaisberger, H., Hartvig, I., Hung, H., Jalonen, R., Kanchanarak, T., Mackay, J., Ping, H., Thammavong, B., Theilade, I., Tran, T., Win, P. and Zheng, Y. (2022). *Dalbergia oliveri*. The IUCN Red List of Threatened Species 2307-8235. Retrieved from *Dalbergia oliveri* (Tamalan) (iucnredlist.org).

Danida (2004). Cambodian Tree Species, pp.6-7.

Fráguas, C. B., Pasqual, M., Dutra, L. F. and Cazetta, J. O. (2004). Micropropagation of fig (*Ficus carica* L.) 'roxo de valinhos' plants. *In Vitro* Cell. Dev-Pl., 40:471-474.

Lloyd, G. and McCown, B. H. (1981). Woody Plant Medium (WPM) a mineral nutrient formulation for microculture of woody plant species. Hort. Sci., 16:453-453.

Murashike, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Journal of Plant Physiology, 15:473-497.

Pradhan, C., Kar, S., Pattnaik, S. and Chand, P. K. (1998). Propagation of *Dalbergia sissoo* Roxb. through in vitro shoot proliferation from cotyledonary nodes. Plant Cell Reports,18:122-126.

Rathnaprabha, D., Muralikrishna, N., Raghu, E., Yashodhara, V. and Sadanandam, A. (2017). Micropropagation of white palash tree (*Butea monosperma* [Lam.] Taub. Var. *lutea* [Witt.]). Journal of Phytology, 9:7-10.

Vibha, J. B., Shekhawat, N. S., Mehandru, P. and Dinesh, R. (2014). Rapid multiplication of *Dalbergia sissoo* Roxb.: a timber yielding tree legume through axillary shoot proliferation and ex vitro rooting. Physiology and Molecular Biology of Plants, 20:81-87.

Supapas, P., Chareonsap, P. P. and Poeaim, A. (2022). *In vitro* micropropagation of siamese rosewood *Dalbergia cochinchinensis* pierre, a timber yielding tree. Pakistan Journal of Botany, 54:1879-1884.

Yarraa, R., Bullea, M., Mushke, R. and Murthy, E. N. (2016). *In vitro* conservation and genetic homogeneity assessment of *Butea monosperma* (Lam.) Taub. Var. lutea (Witt.) Maheshwari—A potential pharmaceutical legume tree. Journal of Applied Research on Medicinal and Aromatic Plants, 195-199.

Winfield, K., Scott, M. and Grayson, C. (2016). Global Status of *Dalbergia* and *Pterocarpus* Rosewood Producing Species. The Convention on International Trade In Endangered Species 17th Conference Of The Parties, Johannesburg, South Africa, pp.61.

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